The Biophysical and Catalytic Characterization of Gpx7 in Presence of Divalent Metal Ions.

FOR PARTIAL FULFILMENT OF
THE MASTER OF SCIENCE DEGREE IN LIFE SCIENCE

2013 - 2015

Submitted by

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DECLARATION

I hereby declare that the thesis entitled **"**The Biophysical and Catalytic Characterization of Gpx7 in presence of Divalent Metal Ions**"**, that I submitted to the Department of Life Science, National Institute of Technology, Rourkela for the partial fulfilment of the Master Degree in Life Science is a record of bonafied and original research work carried out by me under the guidance and supervision of Dr. Suman Jha, Assistant professor in department of Life Science, National Institute of Technology, Rourkela. To the best of my knowledge no part of this thesis has been submitted to any other university or institution for the award of any degree or diploma.

Place- **Bhagyashree Senapati**

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CERTIFICATE

This is to certify that the thesis entitled "Biophysical and catalytic characterization of Gpx7 in presence of divalent metal ions" submitted by Bhagyashree Senapati, roll no.413LS2022 for the award of Master of Science degree from National Institute of Technology, Rourkela is a record of bonafide work, carried out by her under my supervision. Results embodied in this thesis serve to be new and has not been submitted to any university for award of any degree or diploma.

10/15/15

DR. SUMAN JHA

ACKNOWLEDGEMENT

I express my deep sense of gratitude and reverence to my guide, Dr. Suman Jha, Assistant Professor of Life Science, NIT Rourkela, for his valuable guidance and constant supervision throughout this project.

I am extremely grateful and indebted to Miss Kanti Kumari yadav and Mr. Manoranjan Arakha for their inspiring suggestions without which it would have been difficult to carry out this work.

I am highly obliged to Dr. Sujit Kumar Bhutia, HOD of life science department for his suggestion and selection which enables me to find the protein biology laboratory in NIT Rourkela.

Inadequate thought in terms of words and expression, this dissertation owes a lot to my beloved parents and my sister for their support, suggestion, inspiration, encouragement & good wishes for the success of my dissertation.

I may be failing in my duties if I do not thank all my batchmates along with my best friends Kumar Sagar Jaiswal, Gyanaseni Dhar and Shilpa Swagatika Tripathy for their constant encouragement and moral support.

Bhagyashree Senapati

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LIST OF ABBREVIATIONS

1. **Gpx- Glutathione peroxidise.**
2. **GSH- Glutathione.**
3. **2p31- PDB ID for Gpx 7.**
4. **ROS- Reactive oxygen species.**
5. **Ca- Calcium.**
6. **Se- Selenium.**
7. **Zn- Zinc.**
8. **Mg- Magnesium.**
9. **LP- Lipid peroxide.**
10. **µM- Micro molar.**
11. **mM- Mili molar.**

ABSTRACT

Glutathione peroxidase (GPx) is a family of proteins found in biological systems. The protein family is known to detoxicate the system by scavenging reactive oxygen species, formed upon interaction of the system with external moieties or stress conditions or normally synthesized during metabolism of the system. Out of all members of the family, GPx4 and 7 stand alone because of their action on lipid peroxides. GPx4 and 7 are known to reduce lipid peroxides present in lipid membrane into respective lipid alcohol, hence saving the membrane from depolarization. Besides GPx7, other proteins (GPx1-6) of the family are very much known to scientific community. Hence, the underlying mechanism of detoxification by GPx7 is under the scientific scanner. To this end, the thesis has employed extensive *in silico* studies to draw a picture of GPx-7 relations with other proteins of the family, and the possible catalysis efficiency of the protein in presence of different metal ions. In addition, *in vitro* studies (partially) started to validate the catalysis findings from *in silico* studies. The *in silico* findings indicate that cysteine has been re-favored over selenocysteine residue during the evolutionary development of proteins in the family. In addition, the protein share relatively higher propensity for the non-polar environment than other proteins of the family. On catalysis efficiency of the protein, GPx-7 has highest affinity for lipid peroxide in presence of Ca^{2+} ions, and least in presence of Se^{2+}. However, the protein loses its affinity for lipid peroxide in presence of Mg^{2+} or Fe^{2+} ions.

INTRODUCTION

The living eukaryotic cell is comprised of layer bound organelles, which comprises of macro- and micro-molecules consisting of different atoms. Hence, the function depends upon the chemical nature of atoms present in a cell. The chemical behavior of an atom is defined by the number of electrons present in its valence shell. When the valence shell is full, it is quite stable and not engaged in chemical reaction. Whereas unfilled valence shell involves in chemical reaction, hence it is unstable. For gaining its stability, the atom fills the outermost shell by sharing an electron with another atom, hence form a bond. When these weak bonds between electrons are broken, free radicals are formed. Free radicals represent chemically reactive nature because of having odd number of electrons. Hence, free radicals are atoms, molecules or groups of atoms consisting of unpaired electrons present in their outer orbital, which makes them unstable and highly reactive in nature (Cavalcante and Bruin 2009). There are many spontaneous reaction through which free radicals are formed such as exposure to heat, UV light, other factors like smoking, neutralization of viruses and bacteria by immune system, inflammation reaction, air pollution etc., which is figured out in figure 1. Inhuman body, certain essential biomolecules are more susceptible to free radicals such as DNA, RNA, proteins, fatty acids/lipids, vitamins and carbohydrates. Oxygen is very susceptible for free radical formation, and this can be lethal for aerobic organisms.

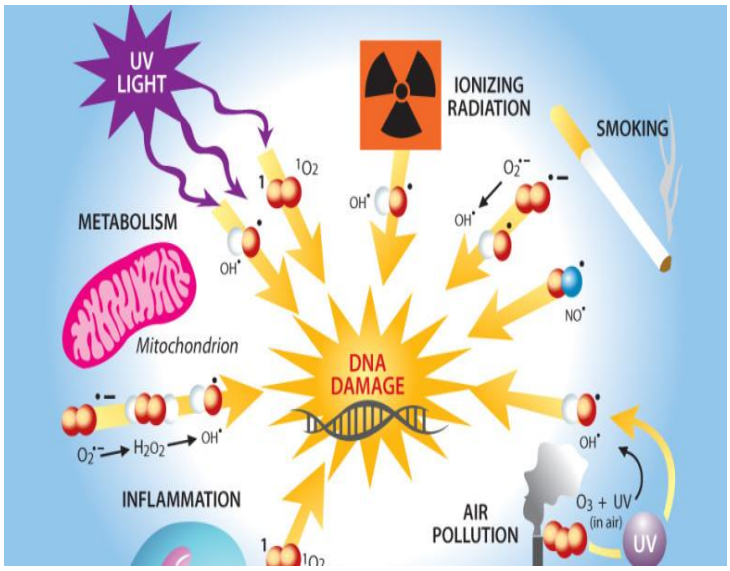


Fig-1. Formation of free radicals through spontaneous reaction.

REACTIVE OXYGEN SPECIES AND IT'S SOURCES

Reactive oxygen species is defined as a term which consisting of both radials and non-radicals oxidizing peroxides (Bayr 2005). Oxygen radicals include superoxide ion, hydroxyl ion, peroxyl and hydroperoxyl etc., and non-radicals oxidizing agents are ozone, hydrogen peroxide, and hypochlorous acid. During cellular metabolism, ROS (reactive oxygen species) is produced and then involved in many enzymatic reactions such as mitochondrial electron transport, activation on nuclear transcription factor, gene expression and antimicrobial action on neutrophils and macrophages. Many enzymatic and non-enzymatic pathways involve and generate reactive oxygen species, which is represented in schematic diagram in figure 2. There are many enzymes such as lipoxygenase, xanthine oxidase, cyclooxygenase, nitric oxide synthase and NADPH oxidase are involved for production of ROS. Organelles involved for generating ROS are mitochondria, endoplasmic reticulum and peroxisome.

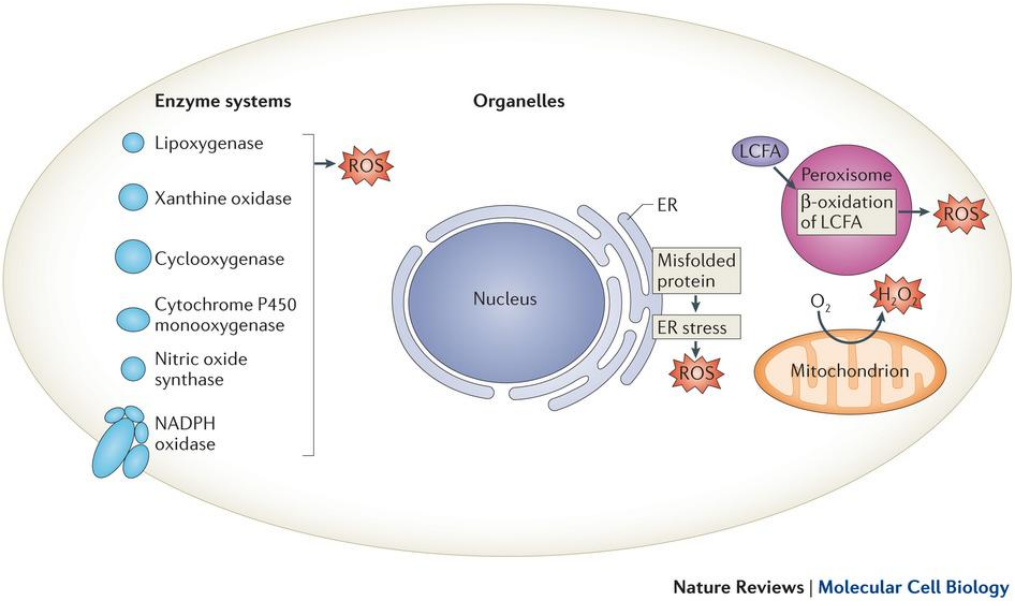


Fig-2 generation of reactive oxygen species(Holmström and Finkel 2014)

It was estimated that 95% amount of oxygen from total amount of oxygen we breath, is concerted tetravalent reduction for producing water by cytochrome oxidase of complex IV in ETC (electron transfer chain) of inner mitochondrial membrane(Cadenas and Davies 2000).An

electron is passed to oxygen directly by generating O_2•−inETC. Mitochondrial production of O_2•− is the major pathway for productionof free radicals in eukaryotic cell. It is estimated that total 160-320 mM of superoxide is produced by a 60 kg woman and 215-430 mM of superoxide is produced by an 80 kg man per day. In addition to inner mitochondrial membrane, mitochondrial outer membrane also involves for the production of reactive oxygen species. An enzyme monoamine oxidase which present in the mitochondrial outer membrane is a large source of H_2O_2 and also catalyzes the oxidative deamination of biogenic amines.

ER lumen is sufficient to activate oxidative stress. During disulfide bond formation, ROS is generated. First, during the transfer of electrons from thiol protein to molecular oxygen which catalyzes by two enzymes such as ERO-1 and PDI, ROS are formed as a by product. Alternatively, at the time of protein misfolding relatively higher amount of ROS is generated due to the depletion of GSH (Tabas and Ron 2011). Due to the repetitive cycles of formation and breakage of disulfide bonds more ROS are generated as a byproduct (Bhandary, Marahatta et al. 2012). Hence, proteins having multiple disulfide bonds may be highly susceptible to generation of more ROS. Accordingly, accumulation of unfolded proteins in the ER inspires Ca^{2+} spillage into the cytosol, expanding ROS generation in the mitochondria(Malhotra and Kaufman 2007).

EFFECT OF ROS

At high ROS concentrations, ROS damages the structure of cells, nucleic acids, lipids and proteins (Jomova, Vondrakova et al. 2010), by bringing chemical modifications in the molecules. The hydroxyl radical ion reacts with all components of DNA molecule. It damages both the purine-pyrimidine bases and also the deoxyribose backbone (Gutteridge and Halliwell 2000).Geneticmaterial's modification resulting from "oxidative damage" represents the initial step involved in ageing, carcinogenesis, mutagenesis.

It is known that generation of ROS through metal inducing process attack DNA as well as other cellular components consisting of polyunsaturated fatty acids, which are highly prone to oxidation (Siems, Grune et al. 1995). Many proteins are oxidized because of ROS. Due to ionizing radiation, some amino acids, simple peptides and proteins are exposed to the oxidation which causes the formation of hydroxyl radicals or a mixture of hydroxyl/superoxide radicals (Stadtman 2004). More specifically side chains of amino acids in proteins such as cysteine and methionine are more susceptible to oxidation due the action of ROS (Stadtman 2004). ROS

involved protein oxidation can be measured by total concentration of carbonyl groups generated by many different mechanisms. For the assay of total protein carbonyl groups, many highly sensitive methods are developed (Dalle-Donne, Giustarini et al. 2003).Many diseases are shown due to ROS induced oxidative stress.

ROLE OF ANTIOXIDANTS

There are series of defense mechanisms develop against free radicals in human body for neutralizing ROS (Cadenas 1997). Defense mechanisms against free radical-induced oxidative stress involve: (i) antioxidant defenses, (ii) preventative mechanisms, (iii) physical defenses, and (iv) repair mechanisms. Any molecule, capable of neutralizing, stabilizing or deactivating reactive oxygen species before cells are attacked by them, is called "antioxidant". There are both enzymatic and non-enzymatic methods involved for neutralizing free radicals which work simultaneously and helps to protect the cell as well as organ system. The antioxidants can be obtained endogenously or exogenously. Non-enzymatic antioxidants involved neutralizing free radicals such as carotenoids, ascorbic acid (Vitamin C), tocopherol (Vitamin E), flavonoids, glutathione (GSH), melatonin, natural flavonoids, and other compounds(Carr, Zhu et al. 2000). Enzymatic defense system of antioxidants includes super oxide dismutase (SOD), glutathione peroxidase (GPX). These are the most efficient enzymatic antioxidants (Strehlow, Rotter et al. 2003).Under certain condition there should be balance between both intracellular levels of antioxidants and their activities. This balance is highly essential for the organism's survival and their health. Among all the antioxidant enzymes, glutathione peroxidase is the most important H2O2 –removing enzymes present in human cell. There are two forms of GPX one is selenium dependent (Gpx, EC1.11.1.19) and other is selenium independent glutathione Stransferase, (GST, EC 2.5.1.18) (Strehlow, Rotter et al. 2003). These differences are seen because of number of subunits, binding of selenium to the active centre and catalytic mechanisms. The most important antioxidative defense mechanism present in the cell is glutathione. Glutathione, a tripeptide is the major thiol antioxidant and redox buffer of the cell (de Freitas, Moreira et al. 2014). There are four different Se-dependent glutathioneperoxidases(Karadag, Ozcelik et al. 2009) and,in total, 8 different GPXs are present in human cell. GPX7 is a type of antioxidant enzyme, which is having cysteine residue instead of seleno-cysteine in active site. It protects cell

from oxidative stress induced by H_2O_2. It also helps in suppressing of bile acid induced ROS generation, and also protects cell from oxidative damage of DNA (Peng, Belkhiri et al. 2012).

The study intend to differentiate the GPX7 from other protein of the family, and help in understanding the mechanism followed by the protein in scavenging of peroxides using both *in silico* and *in vitro* tools.

REVIEW OF LITERATURE

Glutathione peroxidases belong to a protein family which is wide spread over the year and all members of this family have been studied in all kingdom of life (Passardi, Theiler et al. 2007). It was the first seleno enzyme discovered in mammals. Glutathione peroxidase (Gpx) (EC 1.11.1.9) is the name of enzyme family having heme-free thiolperoxidase activity and its main function is rescue the cell from oxidative damage. The main biological role of GPX is to reduce free hydrogen peroxide into water and lipid hydroperoxide into their corresponding alcohol thus diminishing their toxicity (Ursini, Maiorino et al. 1994). Several isozymes are found for GPX gene which varies in their location and substrate specificity. Till date total eight different isoforms (1-8) of glutathione peroxidase have identified in human which is described in table no-1. All are having high sequence similarity, known biochemical function, conserved sesequence pattern, and catalytic triad generally formed by selenocysteine/cysteine, glutamine, and tryptophan (EPP, LADENSTEIN et al. 1983).

Table-1 different isoform of Gpxs in human.

Gene	Locus	Enzyme
GPX1	Chr. 3 p21.3	glutathione peroxidase 1
GPX2	Chr. 14 q24.1	glutathione peroxidase 2 (gastrointestinal)
GPX3	Chr. 5 q23	glutathione peroxidase 3 (plasma)
GPX4	Chr. 19 p13.3	glutathione peroxidase 4 (phospholipid hydroperoxidase)
GPX5	Chr. 6 p21.32	glutathione peroxidase 5 (epididymal androgen-related protein)
GPX6	Chr. 6 p21	glutathione peroxidase 6 (olfactory)
GPX7	Chr. 1 p32	glutathione peroxidase 7
GPX8	Chr. 5 q11.2	glutathione peroxidase 8 (putative)

CATALYTIC MECHANISM OF SEC-Gpxs

The main reaction catalyzes by Gpx is described below.

$$2GSH + H_2O_2 \rightarrow GS–SG + 2H_2O$$

Here GSH is reduced to monomeric glutathione, and GS-SG represents glutathione disulfide.

$$Gpx\text{-}SeH + H_2O_2 \rightarrow Gpx\text{-}SeOH^+ + OH^-$$

$$Gpx\text{-}SeOH^+ + GSH \rightarrow Gpx\text{-}Se\text{-}SG + H_2O$$

$$Gpx\text{-}Se\text{-}SG + GSH \rightarrow Gpx\text{-}SeH + GS\text{-}SG$$

This reaction involves oxidation of the selenol group of selenocysteine by H2O2 which gives the derivative with seleneic acid group. It again converted back to selenol group by a two step reaction in which GSH is involved to form the GS-Se-Gpx and H2O and a second GSH molecule reduces the GS-SeR, a intermediate product to the selenol and releases GS-SG as the by-product(Sies 1993). Gpx kinetics has been identified already decades ago for onlyGpx1 (Ursini, Maiorino et al. 1985) and later found to be similar for Gpx3 and Gpx4 (Takebe, Yarimizu et al. 2002). A ping-pong reaction mechanism is followed by the Gpxs. The total catalytic cycle is divided into the two parts peroxidatic and reductive part which is described in fig- 3. For SecGpx MichaelisMenten kinetics is not described as Vmax and Km are infinite indeed for it. k+1 and k'+2 are rate constant for peroxidatic and reductive steps respectively. First reaction describes by v=k+1[ROOH][Gpxred] and k'+2 describes the two reductive steps. The overall reaction can be defined as d[ROOH]/dt=v= [E0](k+1[ROOH]+k'+2[GSH]). The first GSH forms a selenadisulfide with the selenenic acid and the oxygen isremoved as H2O. The second GSH reduces the selenadisulfide by a thiol–disulfideexchange. Hence GSSG is removed and the enzyme regenerated to the selenol form for further use.

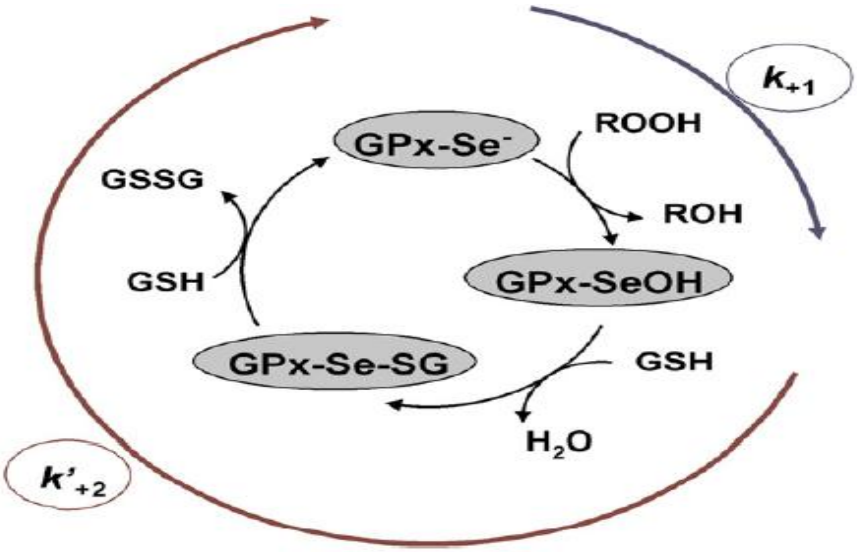


Fig-3 The total catalytic cycle of Gpx

CATALYTIC MECHANISMS OF CYS-Gpxs

The active site Cysteine in CysGpx is located in a NVAxxC(Sec)G motif near the N-terminus. As it is oxidized by the peroxide group to asulfenic acid, hence it is called as peroxidaticCys (Cp), the Cp-SOH in 2 CysGpx reacts with a second cysteine in flexible loop z (Maiorino, Ursini et al. 2007) in the same peptide chain to forming an intramolecular disulfide bond. As second cycle is needed for catalytic cycle, hence it is called as resolving cysteine(CR). The disulfide is

reduced by a thioredoxin with the production of oxidized product Trx (TrxS2). Therefore, these Gpxs are called as thioredoxin peroxidases and their mechanism reflects in a typical 2Cys-peroxiredoxins (Prx)(Flohé, Budde et al. 2003). Sequences of Gpx7 and Gpx8 are CysGpxs without any resolving cysteine hence its reaction mechanism can neither be described as SecGpx.

GLUTATHIONE, AS A REDUCTANT OF Gpx

Most of the selenoproteinsenzyme in mammals use selenocysteine in catalytic site and GSH as a reducing agent. Sec, is the 21st amino acid encoded by an UGA stop codon and recognized by a specific Sec-t-RNA, when a particular stem loop, called selenocysteine insertion sequence (SECIS), forms in the 3'untranslated region (UTR) of the transcript or immediately downstream

region (Walczak, Westhof et al. 1996).GSH is highly abundant in the nuclei (3–15 mM), mitochondria (5–11 mM) and cytosol (1–11 mM) and it is the paramount soluble antioxidant in the mammalian/human cell compartments. By the action of glutamate-cysteine ligase and glutathione synthetase, it is synthesized in the cytosol and a inner membrane transport requires its presence in mitochondria. Two mitochondrial eletro-neutral-antiport carrier proteins such as the dicarboxylate carrier protein and the 2-oxoglutarate carrier protein have shown their capacity to transport GSH. It was also found that externally added glutathione is taken by mitochondria, in spite of approximate 8mM GSH present in the mitochondrial matrix(Shen, Dalton et al. 2005). Hence, GSH is taken up against concentration gradient.GSH maintains the reducing environment of criticalprotein sulphydryls that are necessary for expression and DNArepair. Oxidized glutathione is accumulated inside the cell and the ratio of GSH/GSSG tells about oxidative stress of an organism(Nogueira, Zeni et al. 2004). More concentration of GSSG in the cell may damage oxidatively, many enzymes. GSH plays a major role against oxidative stress(Masella, Di Benedetto et al. 2005) (i) GSH acts as a cofactor of several detoxifying enzyme like glutathione peroxidase, glutathione transferase etc. (ii) it also participates amino acids transport through cell membrane.(iii) GSH scavenges singlet oxygen and hydroxyl radical directly and also helps in detoxify hydrogen peroxide, lipid peroxides by the catalytic action of glutathione peroxidase's catalytic activity. (iv) glutathione enables to regenerate the most important antioxidants to their active forms. The capability of glutathione to revive the most important antioxidants to their active form is linked with the reducing state of the glutathione disulphide-glutathione couple (GSSG/2GSH) (Pastore, Federici et al. 2003).

Redox state of a cell and its movement governs cellular functioning (Schafer and Buettner 2001). The glutathione (2GSH/GSSG couple) is the major cellular redox buffer and for which it is called as an indicator for the redox environment of the cell(Dröge 2003). In many plants, thioredoxin (TRX) acts as a reductant. The intracellular "redox homeostasis" is maintained by GSH/TRX and both maintains the cell signaling process (Dröge 2003). GSH is figured out in following figure-4.

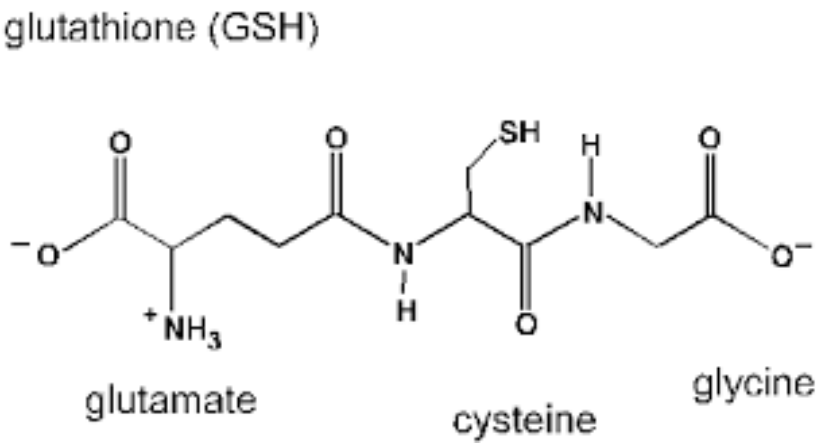


Fig-4 Structure of Glutathione

All mammalian selenoproteins such as Gpx 1-4 having selenocysteine (Sec) in the catalytic centre. In human, only Gpx-6 has Sec in its catalytic center. On the basis of phylogeny, the Gpx family consists of three evolutionary groups arising from a Cys-containing ancestor: Gpx1/Gpx2, Gpx3/Gpx5/Gpx6 and Gpx4/Gpx7/Gpx8 (Herbette, Roeckel-Drevet et al. 2007). All are functioned in a different location of the cell. Gpx1 is ubiquitously present in the cytosol and mitochondria, Gpx2 location is in the intestinal epithelium, Gpx3 present in the plasma, hence all three present in the polar cytosolic condition. Whereas Gpx4 appeared in membrane, and rescue membranous lipid from oxidative damage. Gpx5 having a cys residue was defined as a secreted protein present in the epididymis (Ghyselinck and Dufaure 1990).Gpx6 is only selenoprotein found in human but not in rats or mice (Kryukov, Castellano et al. 2003), and its expression shown in the olfactory epithelium (Dear, Campbell et al. 1991). Gpx7 and Gpx8 have low catalytic activity. For the first time, it has been characterized that Sec or Cys, Gln and Trp present in the catalytic center of Gpxs(EPP, LADENSTEIN et al. 1983) but later it is converted to be a tetrad with an addition of Asn (Tosatto, Bosello et al. 2008). The different functional variations are figured out in the following table-2.

Table-2 Functional variation in all members of Gpx

Mammalian GPx type	Peroxidatic residue	Homo-tetramer	Reducing substrate	Oxidizing substrates
GPx1	Sec	Yes	GSH	H_2O_2, soluble LOOH ROOH Peroxynitrite
GPx2	Sec	Yes	n.d	n.d.
GPx3	Sec	Yes	GSH Low rate with Trx and Grx	H_2O_2, soluble LOOH, ROOH, PLOOH
GPx4	Sec	No	GSH, DTT Mercaptoethanol Cysteine particular protein thiols	H_2O_2, LOOH ROOH, PLOOH ChOOH, CEOOH Peroxynitrite
GPx5	Cys	Yes	n.d.	n.d.
GPx6	Sec in humans, Cys in rats and mice	Yes	n.d.	n.d.
GPx7	Cys	No	GSH, PDI	H_2O_2
GPx8	Cys	No	GSH, PDI?	H_2O_2

Gpx1

Thefirst selenoprotein identified was Gpx1(Rotruck, Pope et al. 1973), and it is also known as the "classical" glutathione peroxidase, now called Gpx-1. Forfirst time it was described as an erythrocyte enzyme that more specifically reduces H2O2 using GSH (Elendt and Bias 1990).It is a homotetramer, reacts with H2O2 and soluble low molecular mass hydroperoxides, such as, cumenehydroperoxide, t-butyl hydroperoxide and hydroperoxy fatty acids (Sies 1993). Later, it was found that the protein also reduces organic hydroperoxides(Christophersen 1969) and even hydroperoxylysophosphatides (Marinho, Antunes et al. 1997). Although it could rescue cell's biomembranes from spontaneous lipid peroxidation in membranes of mitochondria(Flohé and Zimmermann 1970), which ismediated by H2O2, it did not reduce complex lipid hydroperoxide(GROSSMANN and WENDEL 1983).Undersome circumstances when GSH synthetase (GSS) is notavailable such as e.g. in brain mitochondria which do not express GSS, then that case as a reductant for H2O2 Gpx1 can use γ-glutamylcysteine(Quintana-Cabrera,

Fernandez-Fernandez et al. 2012). Gpx1was defined as the enzyme counteracting oxidative stress due to its hydroperoxide reducing capacity.

Gpx2

Gpx2 is a homotetramer protein closely related to the Gpx1. It has high specificity for GSH due to some variation in presence of amino acids such as glutamine and threonine instead of lysine and arginine respectively. Since this protein has not been purified to date thus no catalytic mechanics is addressed for such protein. Among all SecGpxs, Gpx2 ranks highest in the hierarchy followed by Gpx4, Gpx3 and Gpx1 (Wingler, Böcher et al. 1999). Gpx2 is mainly expressed inthe gastrointestinal system more specifically in the epithelium of the oesophagus in humans, also the liver. Therefore, it was also named as GI-Gpx or Gpx-GI, and proposed to act as a defence system against absorption of food-born hydroperoxides(Chu, Doroshow et al. 1993). It is found that highest Gpx2 protein concentrations present at crypt bases and then gradually decline to the top of thecrypts in colon or to the villi in small intestine, respectively(Florian, Wingler et al. 2001). It is observed thatGpx2 mRNA was much higher in extraembryonic tissues in comparison to the embryo itself. The physiological function of Gpx2 turn to opposite if cells are mutated to cancer cell. It was found that Gpx2 is upregulated in colorectal cancer (Al-Taie, Uceyler et al. 2004), Barrett's esophagus (Mörk, Scheurlen et al. 2003), squamous cell carcinoma(Serewko, Popa et al. 2002)or lung adenocarcinomas of smokers (Woenckhaus, Klein-Hitpass et al. 2006). Since activation of the Wnt pathway is characteristic for colon cancer cells, hence the increased expression of Gpx2 might be a consequence thereof.

Gpx3

Like Gpx2, Gpx3 is also similar to Gpx1. It is a tetramer and contains two of the four arginines which is responsible for GSH binding, Arg 103 and 185 (Wingler and Brigelius-Flohé 1999). Rate constant of Gpx3 for hydroperoxides (k+1) are in the range of 10^7 M^{-1} s^{-1}, making Gpx3 a peroxidase as efficient as Gpx1. The reduction rate constant of the selenenic form by glutathione (k'+2) are in the range of 104–105 M−1 s−1 which is a good indicator that GSH acts as a better substrate for Gpx3 at least in vitro.(Takebe, Yarimizu et al. 2002). The reactivity of Gpx3 with thioredoxin and glutaredoxin has been reported (Björnstedt, Xue et al. 1994).Gpx3 is having by a typical N-terminal leader sequence but it lacks an endoplasmic reticulum (ER) retention signal. This suggests that Gpx3 transits the ER and is not retained. Gpx3 is an

extracellular enzyme actively released into the plasma where it is found without leader sequence as a glycosylated protein.

Gpx4

A monomer prtein Gpx4 misses the dimer and tetramer interfaces along with all amino acids involved in GSH binding in Gpx1 (Wingler and Brigelius-Flohé 1999). In spite of the loss of the binding sites, Gpx4 still reacts only with GSH (Ursini, Maiorino et al. 1997) but not with thioredoxin(Takebe, Yarimizu et al. 2002). It had initially been characterized as lipid peroxidation inhibiting protein (PIP) (Ursini, Maiorino et al. 1982) due to its unique ability to reduce, besides H2O2 and small hydroperoxides in general, hydroperoxides in complex lipids such as phospholipid, cholesterol and cholesterol esterhydroperoxides, when they are inserted into cell biomembrane or lipoproteins (Thomas, Geiger et al. 1990). It was observed that Gpx4 can use protein thiol as a reductant instead of GSH, if the GSH amount is depleted,certain cases like chromatin (Godeas, Tramer et al. 1997), sperm mitochondriaassociated cysteine-rich protein (Maiorino, Roveri et al. 2005) and even Gpx4 itself (Mauri, Benazzi et al. 2003). Thus, on the basis of reductant availability it either acts as GSH peroxidase or a thiol peroxidase. Gpx4 present in the cell in three different types of isoforms, a cytosolic (cGpx4), a mitochondrial(mGpx4) and sperm nuclear Gpx4 (snGpx4). All are derived from the same gene which cosisting of 7 exons (Brigelius-Flohé, Aumann et al. 1994). All 7 exons code for the cytosolic and the mitochondrial form, the longer mitochondrial form evolve by an alternative transcription present in the first exon (Pushpa-Rekha, Burdsall et al. 1995). The alternative transcription which present and start in the first codon is used by SnGPX (Maiorino, Scapin et al. 2003) which alternatively leads to the transcription of an exon yielding an N-terminal attachment consisting of a nuclear import signal along with arginine- and lysine-clusters reminding of protamines (Pfeifer, Conrad et al. 2001). It was found that a knockout of cytosolic Gpx4 is the only knockout of a Gpx which proofs as embryonically lethal hence, it seems that Gpx4 is not only an antioxidant but also has some important function which is necessary for life to sustain. Since it is present in the membrane and helps in the oxidization of membrane lipids or lipidhydroperoxide, it might interfere with membrane oxidation. It was observed that in an inducible cGpx4KO mouse system, cGpx4 indeed prevented the activity of 12,15-lipoxygenase (LOX) (Seiler, Schneider et al. 2008). It was shown that 12,15-LOX catalyzes hydroperoxide

formation which acts as a better substrate for Gpx4 and 12,15-LOX triggers apoptosis in some cell.

GpX5

Glutathione peroxidase 5 (Gpx-5), known as epididymal secretory glutathione peroxidase, is an enzyme that in humans is encoded by the *GPX5* gene.Gpx-5 belongs to the glutathione peroxidase family. Its expression is shown in the epididymis in mammal male reproductive tract, and androgen. mRNA of Gpx5 does not contain a selenocysteine (UGA) codon. Thus, the encoded protein is selenium-independent, and it helps to play a role in protecting the membranes of spermatozoa from the damaging effects of lipid peroxidation and preventing premature acrosome reaction. To date, different spliced transcripts variants encoding different isoform of this family have been described (Talmud, Drenos et al. 2009).

Gpx6

Glutathione peroxidase 6 (Gpx-6) is encoded by Gpx6 gene in human.This gene product belongs to the glutathione peroxidase family, which functions in the detoxification of hydrogen peroxide. In its active site,a selenocysteine(Sec) residue is present. The selenocysteine is encoded by the UGA codon, which normally signals translation termination. Expression of this gene is restricted to embryos and adult olfactory epithelium. It is the only selenoproteins in human. It recently studied that this gene is upregulated in the cochleae in mice with age-related hearing loss (Tanaka, Coling et al. 2012)and inplatelet trophoblasts infected with Toxoplasma gondii(Cao, Tao et al. 2013) .

Gpx7

Gpx7 was also known as a novel glutathione peroxidase because of having Cysteine instead of Sec in the catalytic centre in Brca1-null mouse embryonic fibroblasts (Utomo, Jiang et al. 2004). Due to its homology to phospholipid hydroperoxide Gpx4, Gpx7 was named as non-selenocysteinePHGpx (NPGpx). Like Gpx4, NPGpx (Gpx7) is a monomer having a molecular mass of about 22 kDa in SDS-PAGE analysis. Later it was found the location of Gpx7 isin the lumen of endoplasmic reticulum (Nguyen, Saaranen et al. 2011). Gpx7 was marginally expressed in breast cancer cell lines, therefore an inverse relationship between Gpx7 and cancer cell proliferation was found. Some years later, Gpx7 and Gpx3, were found to indeed be down-

regulated in the majority of investigated Barrett's adenocarcinoma samples due to a hypermethylation in their promoter regions(Peng, Razvi et al. 2009).Unthinking studies to investigate the capacity of Gpx7 in oesophageal cells have so far focused on a putative cell reinforcement capacity. By overexpression and down-regulation of Gpx7 in oesophageal epithelial cells, the impact of H2O2- and bile acidmediated intracellular peroxide levels, oxidative DNA harm, doublestrand breaks, apoptosis and oxidation-ward motioning in p38 also, JNK pathways was concentrated on (Peng, Belkhiri et al. 2012). From a diminishing in H2O2 concentration by incubation of equimolar concentration of H2O2 furthermore, recombinant Gpx7 a "peroxidatic" movement has been proposed. Although not demonstrating a peroxidase activity, vanishing of H2O2 may be a sign for alternation of Gpx7 by H2O2 for which a peroxidatic Cysteine target is candidate. Who first described about NPGpx (Gpx7) found Gpx7 to be induced by non-targeting siRNA, which is commonly known as negative signfor controlling of transfection with siRNAfor gene silencing (Wei, Wang et al. 2013). Gpx7 covalently connected with XRN2, an exoribonuclease included in the debasement of mRNA. Effective collaboration of Gpx7 with XRN2 requires an intermolecular disulfide development. The subsequent complex facilitated taking away of accumulated non-targeting siRNA by XRN2(Wei, Wang et al. 2013). As accumulated siRNA is hypothesized to cause oxidation-mediated DNA damage and apoptosis, removal of hydroperoxides is the favoured function of Gpx7 which is a topic of discussion later. It has recently been found that Gpx7 act like a 2CysGpx. However sequence of Gpx7 has characterized that it is the unusual CysGpxwhich is typically equipped with a peroxidaticCys over it (Toppo, Vanin et al. 2008). Gpx7is an enzyme which catalytic mechanism can neither be described as that of a SecGpx or a typical 2CysPrx-like CysGpx and its sequence cannot compile with the possibility that it acts like saccharomyces cerevisiae 2CysGpxtype thiol peroxidase "Orp1", which senses H_2O_2 by being oxidized sulfenic acid form. It also involve in proteins folding. Correct folding pattern including disulfide bond formation is a redox-dependent process that takes place in the reducing environment of endoplasmic reticulum. As newly synthesized proteins is translocated in the reducing environment such as to the ER from cytosol and there they becomes oxidized and further matured by thiol-disulfide bond exchange. During reshuffling of disulfide bond, reducing environment transferred from the protein substrates to oxidized members of the protein disulfide isomerasefamily (PDIs). The thereby reduce PDIs are re-oxidized by ER oxidoreductin-1 (Ero1),

a flavoprotein which transfers the 2 electrons to O2 thus forming H2O2. The H2O2 is used by peroxiredoxin-4 (Prx4) and putatively by Gpx7 and Gpx8 to re-oxidize PDIs(Riemer, Bulleid et al. 2009), Which mechanism is described in the following fig-5.

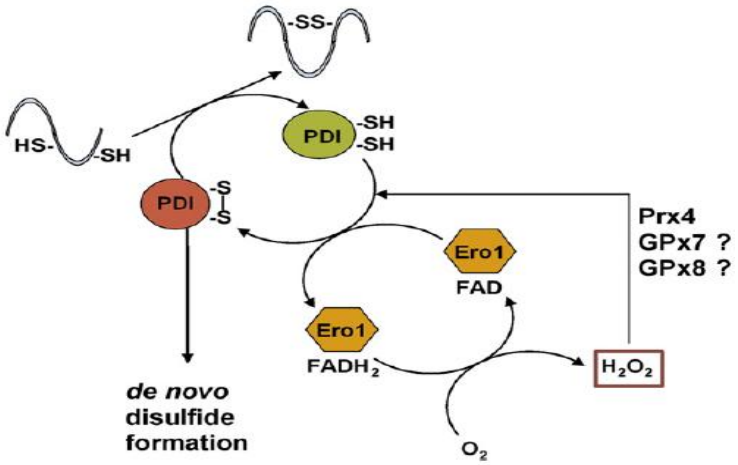


Fig-5 H_2O_2 used by Gpx4, Gpx7 and Gpx8

It was found that this process is associated with production of I molecule of H_2O_2 per disulfide bond, which may proof harm to the body and and also uneconomic. Therefore, the search for utilization of the superfluorous H_2O_2 proved straight forward. Hence ER-resident Prx4 as well

as Gpx7 and Gpx8 were enabled to utilize Ero1-produced H_2O_2 to reoxidize PDI in indeed be able to utilize Ero1-produced H_2O_2 to reoxidize PDI in vitro (Nguyen, Saaranen et al. 2011). It was observed that mice with nonfuctional Ero-1 and truncated were perfectly able to viable (Appenzeller-Herzog, Riemer et al. 2010). Alternative PDI oxidants dehydroascorbate and GSSG, thereby producing ascorbate(Saaranen, Karala et al. 2010), or vitamin K oxidoreductase (VKOR)(Li, Schulman et al. 2010) which drags electrons from reduced PDI and convert it to vitamin K hydroquinone from vitamin K epoxide needed for the γ-glutamyl carboxylation cycle. Also quiescin sulfhydryl oxidases (QSOXs) directly oxidize the protein substrate instead of reduced PDI (Kakihana, Nagata et al. 2012).

Gpx8

Being the last representative detected, it was named Gpx8 and it has been detected as a novel member in a phylogenetic analysis in the Gpx family in mammalia and amphibian (Toppo, Vanin et al. 2008). It is found that Gpx8 is a membrane protein which present in endoplasmicreticulum (Nguyen, Saaranen et al. 2011). However, little is known concerning its function. It is observed that together with Gpx7 it has been suggested to support and helps the reoxidation of PDIs in the folding of proteins. It belongs to the lung-abundant enzymes. Duringinfluenza pneumonia it is decreased and started to recover when tissue started to regenerate (Li, Yin et al. 2013).

OBJECTIVE

In silico

1. To compare the physical, structural properties of Gpx family proteins by SOPMA, PROTPARAM.
2. To find the molecular evolutionary relationship among the family members.
3. To understand the possible efficiency of Gpx7 catalysis in presence of different metal ions.

In vitro

1. Preparation of competent cells and transformation with plasmid containing gene of interest (Gpx7).
2. Optimization of the Gpx7 protein expression.

MATERIALS AND METHODS

In silico studies

Study of structural differences between all members of GPX family

SOPMA

The structure of a protein has a very important role in its function. Every protein has a particular structure. Protein structures are classified into primary, secondary, tertiary, and quaternary. Proteins are synthesized as primary sequence and then it fold to form secondary, tertiary and quaternary structure. Here, using SOPMA, secondary structural identification of proteins was found out. Total four states were found out such as alpha helix, extended strand, beta turn, random coil.

The sequences of all proteins were collected from UNIPROT and then all the information regarding secondary structure were noted out.

Prot-Param

ProtParam is a tool which allows the computation of various physical and chemical parameters for a given protein stored in Swiss-Prot or TrEMBL. The computed parameters include the molecular weight, theoretical pI, amino acid composition, atomic composition, extinction coefficient, estimated half-life, instability index, aliphatic index and grand average of hydropathicity (GRAVY).

Swiss-Prot/TrEMBL accession number (AC) or a sequence identifier (ID) of each protein were collected, then the result of PROT-PARAM was observed.

ClustalW2

ClustalW2 is a general purpose, protein multiple sequence alignment program for three or more sequences. As this proteins belongs to the same family so multiple sequence alignment was done by using ClustalW. All the sequence of proteins were taken from NCBI, and then all sequences were pasted in FASTA format for obtaining the alignment.

Mega4

MEGA is an integrated tool for conducting automatic and manual sequence alignment,inferring phylogenetic trees, mining web-based databases, estimating rates of molecular evolution, and testing evolutionary hypotheses. By using the tool, phylogenetic tree of human Gpxs was drawn to see their molecular evolutionary relationship.

Discovery studio4

Docking was performed using discovery studio4 software. From protein data bank file, proteins structures were downloaded and edited the entire HET atom for obtaining target proteins. Then minimized the structure using charm forcefield, and upon the modifications, proteins structure were derived by prepare proteins tool.

Ligand structures were downloaded using mol2 or molfile,which is having different orientations. Then docking was done, and binding site was defined, hydrogen atom was then added (if it is missing) followed by specifying the protein cavity by CDOCKER(using charmM force field).For docking study some possible combinations were taken for analyzing catalytic efficiency of target protein which is describing below.

2P31_2GSH_ lipid peroxide

2p31_2GSH_Mg_lipid peroxide

2p31_2GSH_Fe_lipid peroxide

2P31_2GSH_se_lipid peroxide

2P31_2GSH_Zn_lipid peroxide

2P31_2GSH_ca_lipid peroxide

From the docking study, we were derived the possible efficiency of Gpx7 catalysis in presence of different metal ions.

In vitro studies

For validating *in silico* observation, we need to express and purify the protein, and do the binding assay to different ligands in presence and absence of metal ions. For the purpose, we proceeded with competent BL21(DE3) bacterial cell preparation, transformation and expression. The steps followed for the work is written in detail in following paragraphs.

BL21(DE3) competent cell preparation

The glycerol stock of BL21DE3 cell was streaked on agar plate to get a single colony, then the single colony was inoculated into 3ml of LB and then allowed for grow over night at 37 degree c at 200 rpm. 1 ml of inoculate was transferred in 250 ml, shaked at 37degreeC for 1.5-3 hours. The OD was checked continuously, when OD reached to 0.3-0.4, then the culture was put on ice for 15 minutes. The cultures were divided into two 50 ml falcon tubes. The cells were then harvested by centrifugation at 6000 rpm for 5 minutes at 4 degree c. About 50 □l of 100 mM $CaCl_2$ was added to each test tube and dissolved the pellet very gently and maked up the volume to 50 ml, then it was incubated on ice for 20 minutes. After 20 minutes, the cells were pelleted down by centrifugation at 6000 rpm for 5 minutes. The pellet was then resuspended in 5 ml of 100 mM CaCl2 along with 30% glycerol and rechilled the microtubes on ice, dispersed 50-500 microlitres of cells in each tubes and freezed at -80 degree c.

Plasmid extraction

Here pNIC28-Bsa4 plasmid was used for transformation and cloning the cell which is described in the following figure-6.

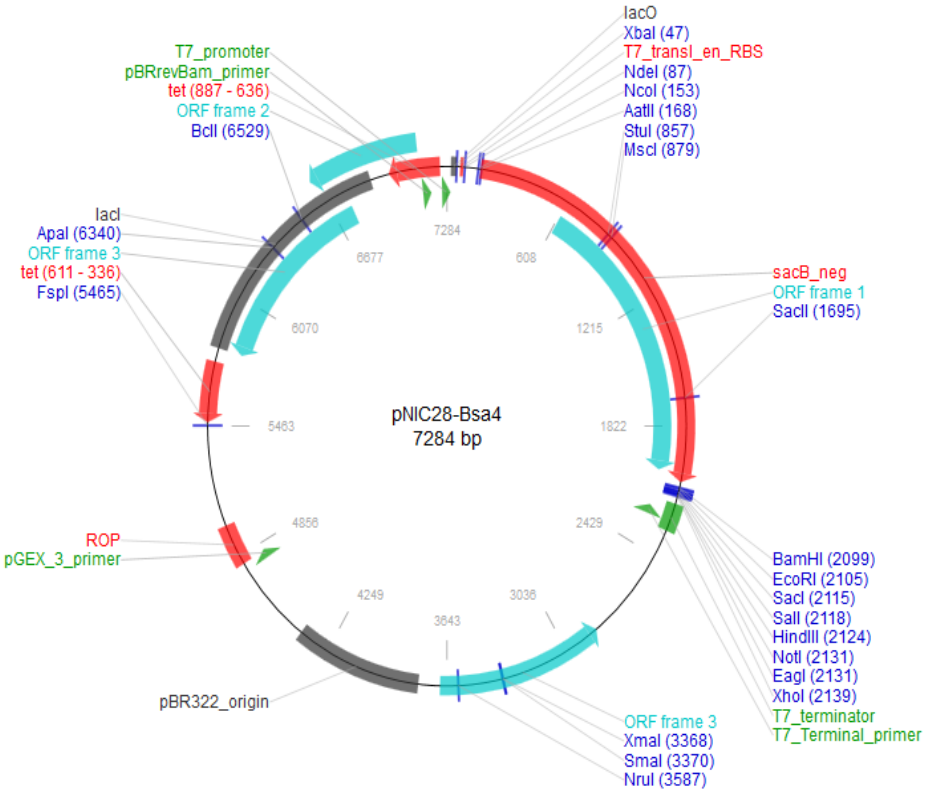


Fig-6 plasmid used for transformation, pNIC28-Bsa4

About 1.5 ml overnight grown culture was poured into microcentrifuge tube, and then centrifuged at room temperature for 60 sec but 12,000 rpm. The supernatant from the tube was completely removed, leaving bacterial pellet as dry as possible. About 100 microlitre ice cold resuspension buffer was added and the bacterial pellet was resuspended properly by vortexing or by slow rounds of pipetting with100 µL containing micro pipette and then incubated on ice for 5 minutes. About 200 µL of freshly prepared lysis solution was added to bacterial suspension. The tube was tightly closed and mixed out thoroughly by incubating and rotting the sealed tube for 4-6 minutes until the solution becoming viscous and slightly clear followed by the incubation on ice for 2-3 minutes. About 150µL of chilled neutralizing solution was added and mixed immediately and thoroughly by incubating and rolling tubes for 4-6 minutes and incubated on ice

for 3-5minutes. The tube was then centrifuged at maximum speed for 10 minutes at 4 degree c. The supernatant containing plasmid was properly transferred in a new centrifuge tube.

Collection of plasmid DNA

Equal volume of phenol: chloroform :isoamylalcoholupto 25:24:1 was added in the supernatant and then mixed by vortexing for 10 sec followed by centrifugation at maximum speed at 4 degree c. The supernantant was then transferred to the microcentrifuge tube. An equal volume of isopropanol was added in the supernatant by incubating the tube up to 4-5 times, centrifuged at maximum speed (14000 rpm) for 30 minutes completely. About 500 micro litre of 70% ethanol was added to the pellet, then the tube was closed and inverted several times followed by centrifugation at 14000 rpm for 5 minutes at 25 degree c. The supernatant was then removed completely. Lastly, the pellet containing plasmid DNA was dissolved in 25 micro litre sterile water or TE buffer. Plasmid concentration was checked.

Insertion of plasmid DNA into the competent cell by artificial chemical transformation

The competent cell was taken out of -80 degree c and thawed on ice for 20-30 minutes approximately. The agar plates containing kanamycin antibiotic were taken out of 4 degree c to warm up to room temperature or placed in 37 degree c incubator. About 1-5 micro litre of plasmid DNA was mixed into 20-50 microlitres of competent cells in a micro centrifuge or falcon tube and then gently mixed by flicking the bottom of the tube by finger a few times. The competent cell/DNA mixture was placed on ice for 20-30 minutes. Each transformation tube was heat shocked by placing the bottom1/2 to 2/3 of the tube into a 42 degree c water bath for 30-60 seconds (45 seconds is usually ideal but this was varied depending on the competent cells). The tube was put back on ice for 2 minutes. About 250-500 µL LB was added and grown in 37 degree c in a shaker incubator for 45 minutes. Some or all the transformants were plated onto a 10 cm LB agar plate containing the appropriate antibiotics, kanamycin. The plates were then incubated at 37 degree celcius overnight.

The transformed colony was collected and cultured over night, after 3-4 hours, IPTG (Isopropyl β-D-1-thiogalactopyranoside) was induced. It worked like an inducer, since it binds at the

repressor site and changed the conformation of lactose repressor hence allowed cell for transformation followed by translation (expression). The untransformed, transformed-induced and transformed-uninduced cells pellets were prepared to check their expressions using SDS-PAGE.

RESULT AND DISCUSSIONS

SOPMA

Table-3 result obtained by SOPMA for all family members of Gpxs:

Serial no.	Name of proteins	% of amino acids involved in α-helix	% of amino acids involved in βstrand	% of amino acids involved in β turn	% of amino acids involved in random coil
1	Gpx1	36.45	17.24	8.87	37.44
2	Gpx2	26.84	23.16	7.89	42.11
3	Gpx3	31.42	23.01	8.85	36.73
4	Gpx4	39.09	23.35	10.66	26.90
5	Gpx5	35.29	20.81	10.86	33.03
6	Gpx6	32.13	27.15	10.41	30.32
7	Gpx7	40.64	22.99	7.49	28.88
8	Gpx8	27.27	27.75	9.57	35.41

From the above data, total % of amino acids contributes for making secondary structures were determined. In brief, Gpx4 and 7 have the highest percentage of ordered secondary structure. The observation indicates the stability of the protein with respect to other proteins. Additionally, α-helix content of both the proteins stands highest than other proteins of the family. The difference in α-helix percentage can be dedicated to their function difference, since both proteins are known to detoxify lipid peroxides present/formed in cellular membrane.

α-helix

For the first time, Pauling and Corey built models of proteins and in addition they found that the alpha helix could be a very stable structure because intra chain hydrogen bonds could be formed that stabilized the helix.

The order of alpha-helical structures were observed among all members of family and ranked as following:

Gpx7>Gpx4>Gpx1>Gpx5>Gpx6>Gpx3>Gpx2>Gpx8.

Hence, from these data it can be concluded that among all Gpx family,Gpx7 and Gpx4 has more amino acids which make alpha helical structure, these are also called as amphoteric amino acids. Due to this it may be indicated their location in the membrane periphery.

β-strand

Although the extended strand is having inter-chain hydrogen bonds it shows the stability of proteins. The role of extended strand is puzzling in proteins. From the above table, the contribution of Gpx family to form the extended strand was observed and ranked as following:

Gpx8>Gpx6>Gpx4>Gpx2>Gpx3>Gpx7>Gpx5>Gpx1

Here, it was observed that although Gpx8 and Gpx6 has highest value to form inter-chain hydrogen bonds but all family members have quite related value in silico study so there is not much difference among them.

β-turn

The β-turn was originally identified in model building studies, by Venkatachalam (1968). Most of these proteins have globular shape, compact structure, requiring reversals in the direction of their polypeptide chains the formation of β-turns plays a major role in molecular recognition, detecting protein stability and protein folding. Proline and glycine are frequently present in β-turn. Hence, it may be involved for making disorder structures. From the above table, the following data were observed.

Gpx5>Gpx4>Gpx6>Gpx8>Gpx1>Gpx3>Gpx2>Gpx7

From the observed data it may be concluded that among all family,Gpx7 has less number of β-turn value. As Gpx7 does n't have more number of structural breakers (helix breaker), it may provide strong evidence against the most stable helical structure of it. Among all Gpx5 has highest ability to form β-turn. Hence, it helps in formation of loop.

Random Coil

Monomer subunit are structured randomly and forms this structure. The conformation's name is derived from the idea that, in the absence of specific, stabilizing interactions, a polymer backbone will "sample" all possible conformations randomly. From the above data, rank order for forming random coil was observed which is described in following:

Gpx2>Gpx1>Gpx3>Gpx8>Gpx5>Gpx6>Gpx7>Gpx4

From observed data, it was clearly indicated that among all family, Gpx7 and Gpx4 are having less value to form random coil. Hence it may be concluded that Gpx7 and Gpx4 are very stable due to having strong stabilizing interaction among them.

Prot-param

By using prot-param, the physical parameter of all member of Gpx family were observed and compared among them.

Table-4: Result obtained by prot-param for all member of Gpx family.

S. no.	Name of proteins	Molecular weight of proteins (Dalton)	Total number of a.a.	Total number of –vely charged a.a.	Total number of+vely charged a.a.	Theoretical pI
1	Gpx1	22088.1	203	21	20	6.15
2	Gpx2	21954.0	190	22	23	7.64
3	Gpx3	25552.3	226	23	25	8.26
4	Gpx4	22174.6	197	21	26	8.69
5	Gpx5	25202.2	221	23	28	8.83
6	Gpx6	24970.5	221	20	18	6.21
7	Gpx7	20996.0	187	18	20	8.42
8	Gpx8	23881.0	209	23	33	9.41

Molecular weight

The *molecular weight* of each family member were observed and compared as following

Gpx3>Gpx5>Gpx6>Gpx8>Gpx4>Gpx1>Gpx2>Gpx7

Hence, it was observed that Gpx3 has highest molecular weight among all members of family and Gpx7 has least molecular weight among all members of family. *Total number of amino acids* for each family member was observed and compared as following:

Gpx3>Gpx5/Gpx6>Gpx8>Gpx1>Gpx4>Gpx2>Gpx7

From the above table, total *number of negatively charged amino acids* present in each members of Gpx family was observed and compared as following:

Gpx3/Gpx5/Gpx8>Gpx2>Gpx1/Gpx4>Gpx6>Gpx7

Total *number of positively amino acids* present in each members of Gpx family was observed and compared as following: *Gpx8>Gpx5>Gpx4>Gpx3>Gpx2>Gpx1/Gpx7>Gpx6*

Hence Gpx8 has highest numbers of positively charged amino acids, which indicates that it may have higher theoretical pI.

Theoretical pI

The isoelectric point(pI/pH(I)/IEP), is the pH at which a particular molecule carries no charge, i.e. they exist in zwitterionic form. The pI value can affect the solubility of a molecule at a given pH. Such molecules have minimum solubility in water or salt solutions at the pH that corresponds to their pI and often precipitate out of solution. The protein having more pI value means it is having more positively charged amino acids. The observed data was compared as following for the pI value:

Gpx8>Gpx5>Gpx4>Gpx7>Gpx3>Gpx2>Gpx6>Gpx1

Positively charged amino acids at physiological pH indicate that the residue is basic. Hence, the protein with highest number of such residue will have relatively higher pH value at which the protein will exist as zwitterion form. Thus, Gpx8 has highest pI value because of highest number of basic residue in its sequence.

Table-5:Result obtained by Prot-param software for all members of Gpx.

S. no.	Name of proteins	Instability index	Aliphatic index	Grand average hydropathy	Estimated half life	Extinction coefficient
1	Gpx1	47.96	86.11	-0.070	20hrs (yeast) 10 hrs(*E.coli*)	0.779
2	Gpx2	48.91	82.05	-0.320	20hrs (yeast) 10 hrs(*E. coli*)	0.988
3	Gpx3	53.55	83.23	-0.221	20hrs (yeast) 10 hrs(*E. coli*)	1.239
4	Gpx4	31.83	79.75	-0.194	20hrs (yeast) 10 hrs(*E. coli*)	1.418
5	Gpx5	48.73	83.26	-0.285	20hrs (yeast) 10 hrs(*E. coli*)	1.197
6	Gpx6	33.50	81.09	-0.096	20hrs (yeast) 10 hrs(*E. coli*)	1.088
7	Gpx7	42.29	85.03	-0.136	20hrs (yeast) 10 hrs(*E. coli*)	1.742
8	Gpx8	32.74	93.21	-0.136	20hrs (yeast) 10 hrs(*E. coli*)	1.076

Instability index

The instability index provides an estimate of the stability of a protein in a test tube. Statistical analysis of 12 unstable and 32 stable proteins has revealed that there are certain dipeptides, the

occurrence of which is significantly different in the unstable proteins compared with those in the stable ones. A method was obtained to predict the instability index value which is defined as:

$$II = \frac{10}{L} * \sum_{i=1}^{i=L-1} DIWV(x[i]x[i+1])$$

where L is the length of sequence

DIWV(x[i]x[i+1]) is the instability weight value for the dipeptide starting in position. From the above data, it is found that only 3 members of this family have stable instability complex and these are Gpx4, Gpx6 and Gpx8.

Aliphatic index

The aliphatic index of a protein is determined as the volume covered by aliphatic amino acids side chain. It may be regarded as a positive factor for the increase of thermostability of globular proteins. From the above data, following comparisons were figured out: *Gpx8>Gpx1>Gpx7>Gpx5>Gpx3>Gpx2>Gpx6>Gpx4*

Hence it was concluded that Gpx8 has highest value for aliphatic index. Target protein of interest Gpx7 has also quite high value of aliphatic index which hypothesizes that it may present in the periphery of the lipid membrane.

Grand average of hydropathicity

Hydropathicity is defined as the relative hydrophobicity or hydrophilicity of a compound, especially of an amino acid residue in a protein. The GRAVY (grand average hydropathy) value for a peptide or protein calculates as the sum of hydropathy values of all the amino acids, divided by the total number of residues in the sequence. From the prot-param data, it was observed that all the members of this family have the negative value which represents that these are having more number of hydrophilic aminoacids.

Gpx1>Gpx6>Gpx7/Gpx8>Gpx4>Gpx3>Gpx5>Gpx2

Hence, it was observed that among all family members, Gpx1 has highest value for GRAVY (grand average hydropathy). Gpx7 has also quite higher value than others which represents that it is having more number of aliphatic aminoacids, and helps to make present on membrane.

Estimated half life

The half-life is defined as time taken for disappearance of half protein population after synthesis in cell. ProtParam depends upon the "N-end rule", which relates the half-life of a protein to the identity of its N-terminal residue, the prediction is given for 3 model organisms (human, yeast and *E.coli*). The N-end rule originated from the observations that the identity of the N-terminal residue of a protein plays an important role in determining its stability *in vivo*. It was observed that all the proteins of Gpx family have same estimated half-life which is described below.

The N-terminal of the sequence considered is M (Met).

The estimated half-life is: 30 hours (mammalian reticulocytes, *in vitro*).

>20 hours (yeast, *in vivo*).

>10 hours (*Escherichia coli, in vivo*).

Extinction coefficients

Extinction coefficient refers to several different measures of the absorption of light in a medium. It determines light absorption by a substances in a particular wavelength. More extinction coefficient represents the more no of aromatic amino acids and cysteine.

From the prot-param data it was observed like below.

Gpx7>Gpx3>Gpx4>Gpx5>Gpx6>Gpx8>Gpx2>Gpx1

It is interesting to find that Gpx 3 and 5, although, have highest number of amino acids, but Gpx7 has highest number of residues that absorbs at 280 nm. Hence, Gpx7 has the highest extinction coefficient among the family members.

Clustal W

By using clustalW, whole sequences were aligned to detect the conserved sequence among them and total % of similarity in their sequence were identified, which is described in figure-6.

Fig-7 Result obtained by ClustalW.

By using clustalw, all the sequences from each member of Gpx are aligned. From aligned data conserved sequence among them was observed below which * marks were found in the figure. The obtained conserved sequences in Gpx7 were from 83 to 89. In active site, selenocysteine present at 49 position, 40 position, 73 position in Gpx1, Gpx2, Gpx3, Gpx4 and Gpx6 respectively. Active site position of cysteine present in 73 position, 57 position and 79 position in Gpx5, Gpx7 and Gpx8 respectively.

Mega4

From the Mega4, data the evolutionary relationship among the family was analysed, and drawn the family tree in the following figure:

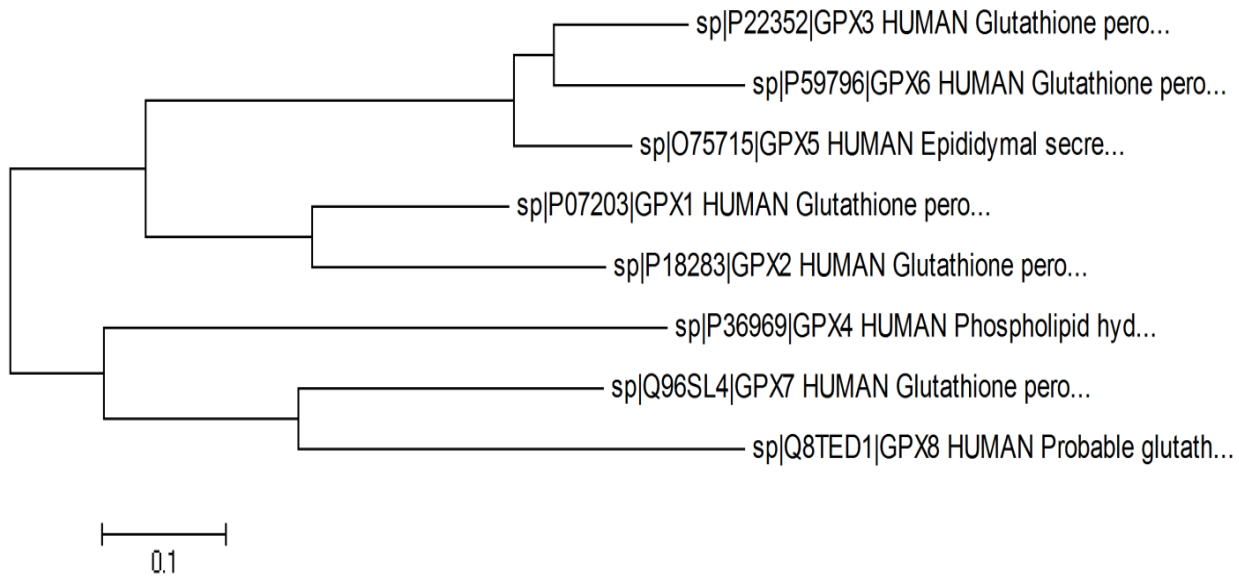


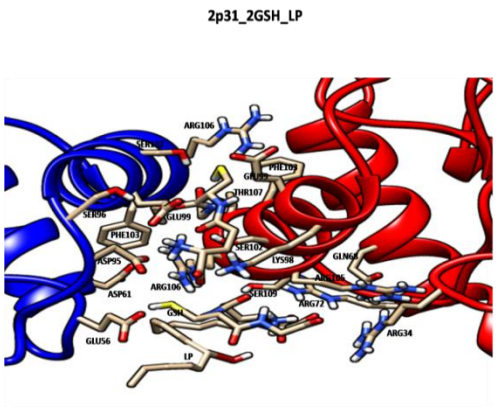
Fig-8: Evolutionary relationship among all members of Gpxs in human.

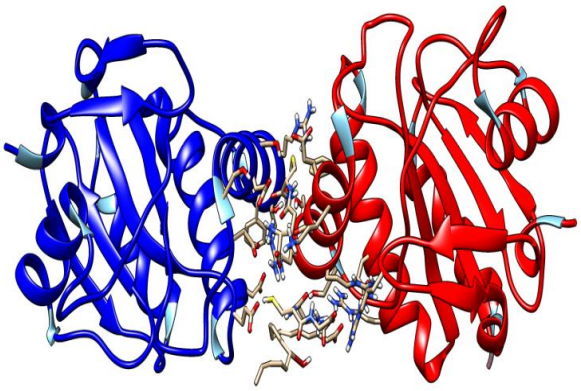
From the above figure 7, it was observed that Gpx7 and Gpx8 evolved from the same parents and they had very close proximity to Gpx4 than other member of the family. Hence, it is hypothesized that they may share similar functional property. Gpx1 and Gpx2 were evolved from the same ancestor who shares its ancestor with the ancestor of Gpx5, 3 and 6. However, Gpx3 and Gpx6 evolved from same ancestor which gave root to Gpx5.

Docking study

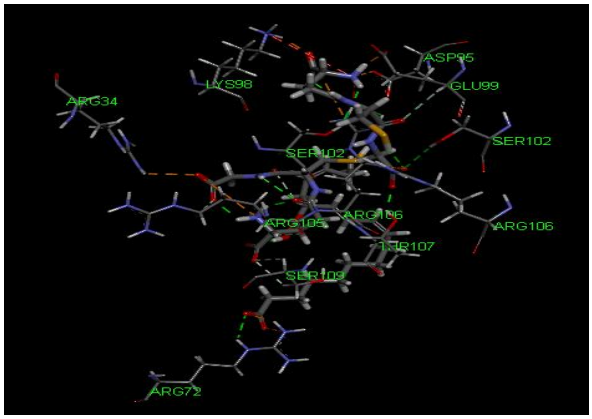
Docking is a tool which helps to determine the orientation of one molecule when it bound to second molecule and helps to form a stabilized complex. This stabilization was calculated by calculating the binding energy between two molecules. We designed 6 sets of experiments for observing the experiments to check the possible catalytic efficiency of Gpx7 in presence of metal ions such as Ca, Zn, Se, Fe and Mg ions due to their availability in eukaryotic cell. As it is found from literature that Se is not involved for catalytic active site of Gpx7 so it was taken to check the efficiency of Gpx7 in the presence of Se ion. By using visualization tool such as chimera, discovery and superimposed all possible interaction were observed and binding energy were noted down and discussed in following figure-9.

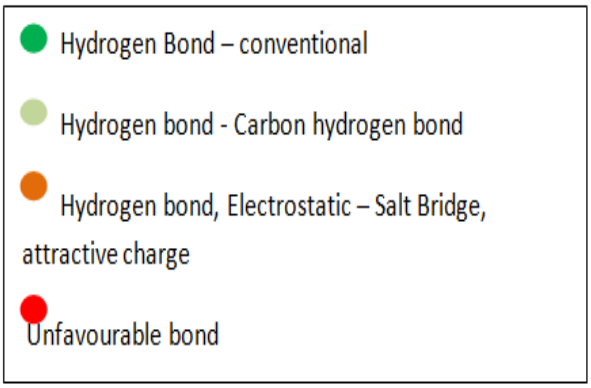
2p31_2GSH_Lipid peroxide





9(a).chimera 9(b).superimpose



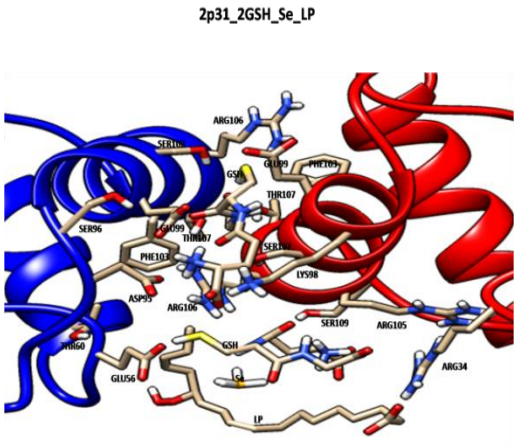


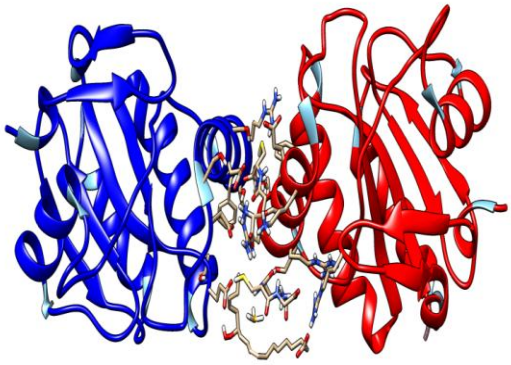
9 (c) discovery

Figure 9. Binding energy of different 2p31_2GSH_Lipid peroxide

From above figures, it was observed that the binding energy of Gpx7 (2p31) in the presence of GSH and lipid peroxides is -77.5681 Kcal/mol. Hence, it was concluded that the efficiency was quite less for detoxifying lipid peroxide in presence of 2GSH. Hence as we were supposed to have more efficiency of Gpx7 in presence of metalions, docking was further proceeded to check the efficiency. The residue which interacts with water part of ligand by hydrogen bonds which is shown as green in color.

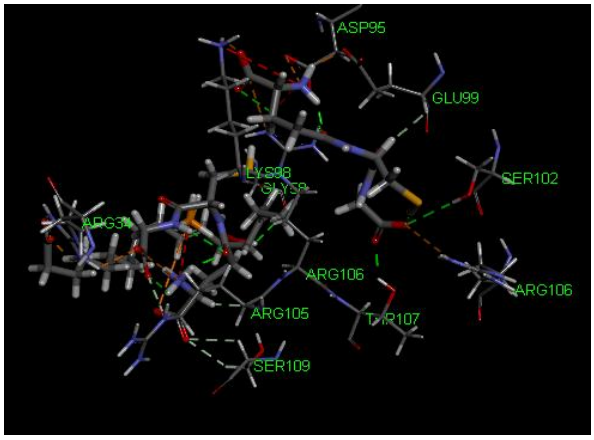
2p31_2GSH_Se_lipid peroxide





10.(a)chimera

10.(b)superimposed

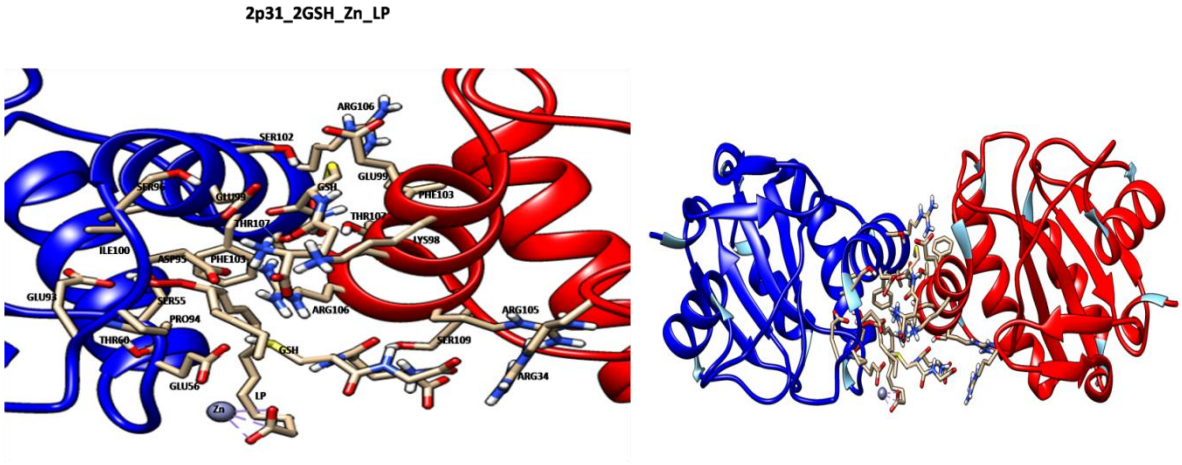


10.(c) discovery

Figure 10. Docking study of 2p31_2GSH_Se_lipid peroxide

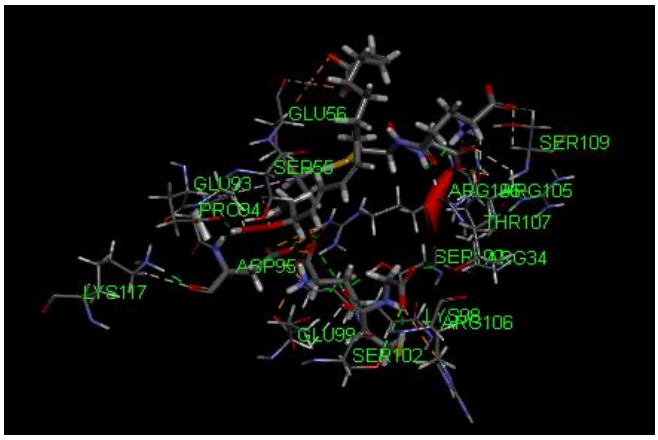
Here 2p31 was first allowed to form a complex with 2GSH, then with Se and lipid peroxide respectively. From the doscovery tool, binding energy was calculated for it and found as -72.34 Kcal/mol, hence it was assumed that it may not be acted as a good enhancer for increasing catalytic efficiency of Gpx7 by comparing with binding energy of Gpx7 with 2GSH and lipid peroxide. The ligand which interacts with water part shown in green in color in discovery figure.

2p31_2GSH_Zn_lipidperoxide



11.(a)chimera

11.(b)superimposed

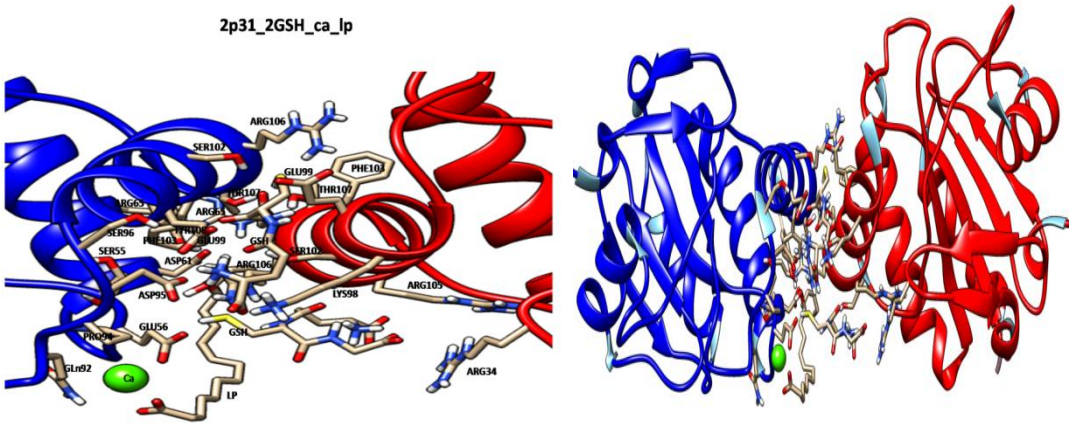


11.(c) discovery

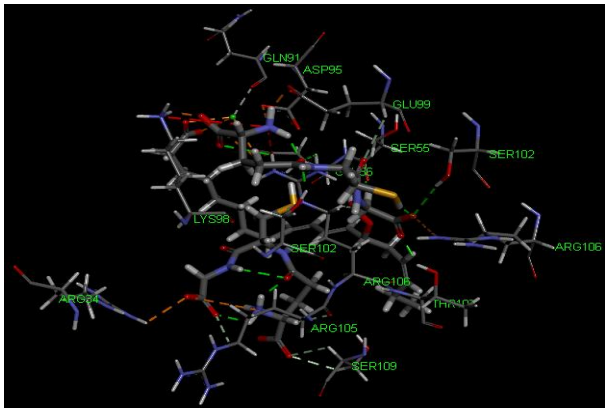
Figure 11. Docking study of 2p31_2GSH_Zn_lipidperoxide

Here 2p31 was first allowed to form a complex with 2GSH, then with Zn and lipid peroxide respectively. From the doscovery tool, binding energy was calculated for it and found as -115.932, hence it was assumed that it may be acted as a good enhancer for increasing catalytic efficiency of Gpx7 by comparing with binding energy of 2p31_2GSH_lipid peroxide and 2p31_Zn_2GSH_lipid peroxide.

2p31_2GSH_Ca_lipid peroxide



12.(a)chimera 12.(b)superimposed



12.(c) discovery

Fig-12. Docking study of 2p31_2GSH_Ca_lipid peroxide

Here, 2p31 was first allowed to form a complex with 2GSH, then with Zn and lipid peroxide, respectively. From the doscovery tool, binding energy was calculated for it and found as -208.17Kcal/mol, hence it was assumed that it may be acted as a good enhancer for increasing catalytic efficiency of Gpx7 by comparing with binding energy of 2p31_2GSH_lipidperoxide and 2p31_Zn_2GSH_lipid peroxide.

2p31_2GSH_Mg_lipid peroxide

The docking of lipid peroxide (substrate) to 2p31_2GSH_Mgcomplex failed, which indicates that the lipid peroxide can not bind with the complex. There can be two reasons for substrate not able to bind the complex are: (i) docking of Mg ion to 2p31_2GSH complex is disorienting the binding site, making it unavailable to bind with the substrate, and/or (ii) Mg ionis binding to active site of the complex. To rationalise the reasons, superimposition of the Mg dockedprotein was done with undocked protein, and the result is represented in fig. 13

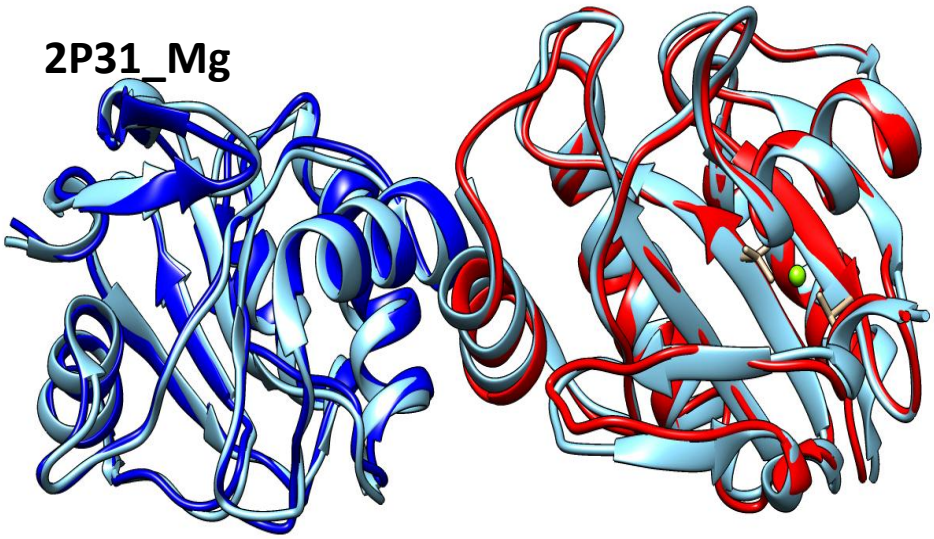


Fig-13: shows Superimposition of 2p31_Mg conformation with 2p31 conformation.

From the above figure-13, it was found that the structure of active side is changing upon Mg ion docking to the protein. Hence, it may be concluded that due to the structural disorderness, substrate may not able to bind with the complex in presence of Mg ion.

2p31_2GSH_Fe_lipid peroxide

Like Mg ion, the docking of lipid peroxide (substrate) to 2p31_2GSH_Fe complex failed, which indicates that the lipid peroxide can not bind with the complex.

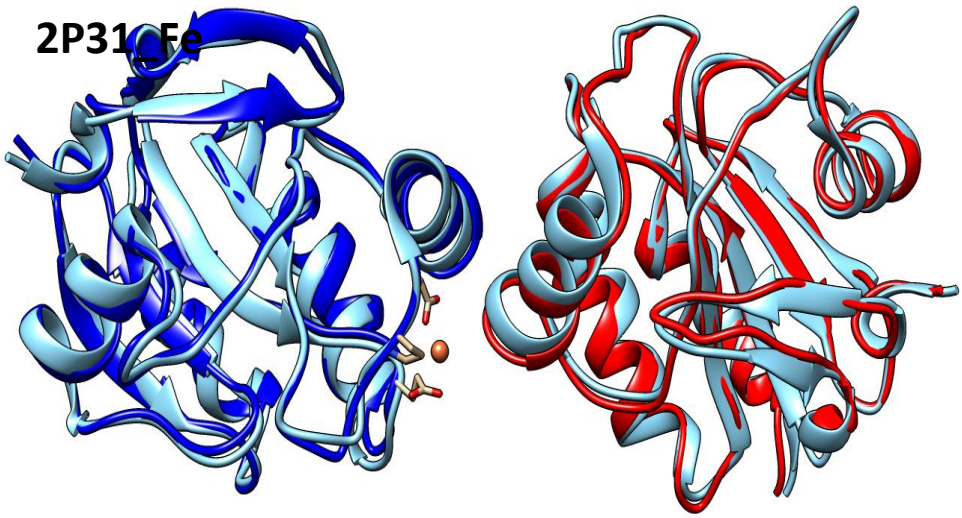


Fig-14 super imposed structure of 2p31_Fe

The superimposition data indicate that the presence of Fe ion brings the structural disorderness in the protein which is vital for substrate binding to the complex.

Comparison of substrate binding to the complex from binding energy point of view is:

2p31_2GSH_Ca_lipidperoxide > 2p31_2GSH_Zn_lipidperoxide>2p31_2GSH_Se_lipidperoxide.

From the docking study, it is observed that lipid peroxide binding to Gpx7(2p31)_2GSH complex result in release of binding energy of -77.5681 Kcal/mol. However, presence of the metal ions in the complex, except Fe and Mg ions, result in many fold increase in binding energy of lipid peroxide to the complex. Hence, it is highly possible that the presence of Ca, Zn, and Se ions may enhance the efficiency of the protein function in physiological conditions.

To validate the possibility of the increase in efficiency of the protein, next phase of the thesis involved transformation and expression of the protein in bacterial system, *E. coli* BL21(DE3) strain.

In vitro study

Protein expression

The protein expression was checked through SDS-PAGE. From the protein bands, expression was observed by analyzing the thickness of bands which was figured out below in figure-15.

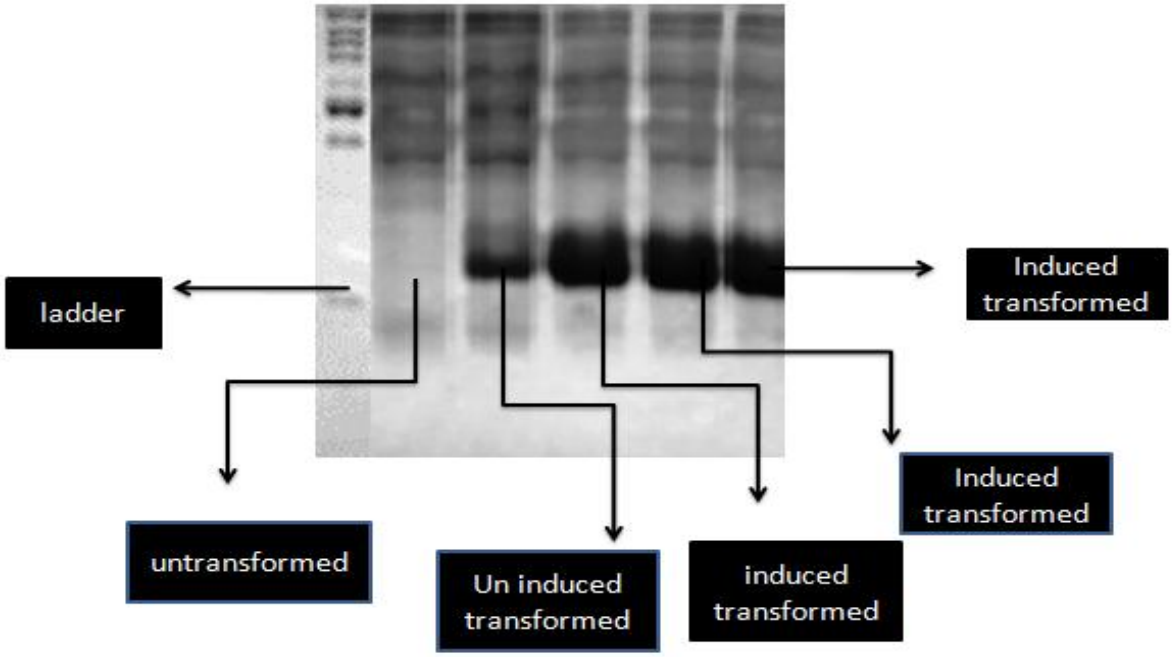


Fig-15 shows Gpx7 protein expressions

From the above figure, expression of target protein was observed. The protein expression upon transformation with the plasmid and induction with IPTG shows higher level of expression. The enhanced expression was confirmed from elevated intensity of staining dye corresponding at 20 KDa band. Un-induced culture also showed band at 20 KDa, but the intensity of staining dye is less than that of induced band. Un-transformed culture did not show any band corresponding to the desired molecular weight protein.

Hence, it was concluded that the protein was over-expressed in presence of an inducer such as IPTG

.

CONCLUSION

The structural and physical properties of Gpx7 with other member of proteins were compared and analyzed which gives a brief idea about biophysical nature of the nascent Gpx7. Our protein of interest Gpx7 has high intra-chain hydrogen bonds, and it has higher content of amino acids which involves in helix formation. It has quite less amount of amino acids which participates in extended strand formation. It has less number of amino acids which involves in forming β-turn. Hence, quite less number of helix breakers present in the sequence. It has very less number of amino acids which involves in random coil, so it is having most stable structure among the family members.Its physical properties were also found out which helps to relate the functional properties of Gpx7 with other proteins of the family. Evolutionary relationship among all family members was observed, and their structural as well as functional properties of related groups were found out. It shows the presence of cysteine residue in catalytic active site and is highly conserved like other residue of active quadrant.

catalytic activity of Gpx7 was checked in presence of metal ions and it was found that in the presence of calcium its efficiency for detoxifying lipid peroxide gradually increases. In vitro analysis was done by checking the expressions of Gpx7 proteins

As *in silico* study helps for obtaining prior knowledge to gain expertise in doing the same work *in vitro*. Hence, it acts as the pillar work against Gpx7 for more future work.

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